PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference L 17-18088.1/me	**FOR FURTHER ACTION**	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
International application No. PCT/EP 03/08308	International filing date *(day/month/year)* 28.07.2003	Priority date *(day/month/year)* 31.07.2002
International Patent Classification (IPC) or both national classification and IPC C07K14/535		
Applicant LEK PHARMACEUTICALS D.D. et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 9 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 3 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the opinion
   - II ☐ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☒ Lack of unity of invention
   - V ☒ Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☐ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
23.01.2004	30.09.2004
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized Officer Rojo Romeo, E Telephone No. +49 89 2399-7321

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/EP 03/08308

I. Basis of the report

1. With regard to the **elements** of the international application *(Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17))*:

Description, Pages

1-24 as originally filed

Claims, Numbers

1-24 received on 15.09.2004 with letter of 14.09.2004

Drawings, Sheets

1/5-5/5 as originally filed

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

   These elements were available or furnished to this Authority in the following language: , which is:

   ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).

   ☐ the language of publication of the international application (under Rule 48.3(b)).

   ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

   ☒ contained in the international application in written form.

   ☒ filed together with the international application in computer readable form.

   ☐ furnished subsequently to this Authority in written form.

   ☐ furnished subsequently to this Authority in computer readable form.

   ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

   ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

   ☐ the description, pages:

   ☐ the claims, Nos.:

   ☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)).

   (Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

IV. Lack of unity of invention

1. In response to the invitation to restrict or pay additional fees, the applicant has:

   ☐ restricted the claims.

   ☐ paid additional fees.

   ☐ paid additional fees under protest.

   ☐ neither restricted nor paid additional fees.

2. ☒ This Authority found that the requirement of unity of invention is not complied with and chose, according to Rule 68.1, not to invite the applicant to restrict or pay additional fees.

3. This Authority considers that the requirement of unity of invention in accordance with Rules 13.1, 13.2 and 13.3 is

   ☐ complied with.

   ☒ not complied with for the following reasons:

   see separate sheet

4. Consequently, the following parts of the international application were the subject of international preliminary examination in establishing this report:

   ☒ all parts.

   ☐ the parts relating to claims Nos. .

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1, 8, 9, 13, 14, 16, 17, 21, 22
	No:	Claims	2-7, 10-12, 15, 18-20, 23, 24
Inventive step (IS)	Yes:	Claims	
	No:	Claims	1-24
Industrial applicability (IA)	Yes:	Claims	1-24
	No:	Claims	

2. Citations and explanations

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/EP 03/08308

see separate sheet

Re Item I

Basis of the report

Concerning the following comments, Applicant's arguments were carefully considered but were not found to be convincing.

Re Item IV

Lack of unity of invention

Applicant's attention is drawn to the fact that, in view of the cited prior art, silent mutants of hG-CSF were already known which improved the expression of hG-CSF in E. coli and had the technical features claimed. Consequently, an objection for lack of unity is to be expected in the regional phase.

Re Item V

Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

Reference is made to the following documents cited in the International Search Report:

D1: Gene, Elsevier Biomedical Press. Amsterdam, Nl (1988), 65(1), 13-22
D2: US-A-5840543
D3: Protein Expression And Purification (11-2001), 23(2), 311-318
D4: Current Opinion In Biotechnology, London, Gb (10-1995), 6(5), 494-500
D5: Microbiol. Rev. (01-09-1996), 60(3), 512-538

The present application concerns synthetic genes coding for the human granulocyte-colony stimulating factor (hG-CSF) which enables an expression in E. coli of 52% or more of total protein. The mutant described (no changes at the amino acid sequence level) has changes in the 5' first 194 nucleotides.

The synthetic gene encoding hG-CSF of the present invention is constructed by the combination of the following methods:

- replacement of E. coli rare codons with E. coli preference codons: in the segment II (between restriction sites SacI (194) and ApaI (309) and in the segment IV (between restriction site NheI (467) and BamHI (536),
- replacement of GC rich regions with AT rich regions, whereby the rarest E. coli codons are replaced, but mostly not with the E. coli preference codons: in the segment I (between restriction sites NdeI (3) and SacI (194),
- completely unchanged native sequence of 46 codons (between CCC for Pro 102 and CGC for Arg147) in the segment III,
- replacement of two E. coli rare codons (CGG->CGT (Arg148) and GGA->GGT (Gly150)

at the terminal end of the segment III.
The mutant of SEQ ID NO: 1 carries all the mutations disclosed at pages 9 and 10 of the present application.

1. Novelty (Art. 33(2) PCT)
Applicant's attention is drawn to the fact that claim 2 was interpreted as being directed to a DNA sequence encoding a variant of hG-CSF characterized in that its sequence comprises a combination of modifications as listed in claim 2. The present wording of claim 2 is, however, unclear, a nucleotide sequence not being able to comprise a nucleotide sequence selected from a list of modifications (a modification is not a nucleotide sequence).
In addition, a "combination of the modifications" does not imply that all the modifications need to be present, a combination of two of them being sufficient.

1.1 Document D1 discloses a variant of hG-CSF bearing silent mutations decreasing the G + C content of the 5' end of the coding region of this gene (see e.g. Fig. 1). This variant can be viewed as a sequence comprising a partial sequence of SEQ ID NO: 1 and it comprises modifications in segment I as defined in the present application. The variant of D1 can thus be viewed as representing a combination of variations in segment I and no changes in segment III. D1 is thus novelty destroying for the subject-matter of claims 2, 3, 5, 6, 7, 10, 11, 12, 15, 18, 20, 24.
The same teaching can be derived from documents D2 and D3.

1.2 In addition, D2 discloses variants of hG-CSF bearing silent substitutions as taught by the present application (Fig. 6 and 7). For instance, the variant of pICI 1056 bears 32 of the exact silent substitutions taught by the present application at pages 9 and 10 and 13 silent substitutions at positions also taught by the present application (Fig. 6). These substitutions are distributed over the entire hG-CSF sequence, i.e. in the 4 different segments as defined at pages 7 and 8 and claim 2 of the present application. The constructs described in this document led to an expression of hG-CSF between 40% and 50% of total E. coli protein (see Fig. 3A and 4A). This document is novelty destroying for the subject-matter of claims 2-7, 10, 11, 12, 15, 18-20, 23, 24.

1.3 Moreover, claims 4, 19 and 23 are drawn as results to be achieved without providing any technical feature which should lead to the claimed technical effect. Moreover, in the absence of reference (an expression level of at least 50% of total E. coli

proteins?), these claims are unclear. An objection for lack of novelty in view of D1-D3 is also raised for this reason.

Concerning this, applicant's attention is drawn to the fact that the added wording "to the total proteins after expression, of at least 50%..." does not clarify the lack of reference pointed out above.

In summary, claims 2-7, 10, 11, 12, 15, 18-20, 23 and 24 are not novel.

1.4 Due to the massive clarity problems of the current set of claims, Applicant is recommended to have the claims reworded by an English speaker before entering the European regional phase.

2. Inventive step (Art. 33(3) PCT)

2.1 In view of document D2, the subject-matter of claims 8, 9, 13, 14, 16, 17, 21, 22 are obvious embodiments of the claims which are not novel and thus, do not show any inventive activity.

2.2 The document D2 is regarded as being the closest prior art to the subject-matter of claim 1, and discloses variants of hG-CSF bearing silent substitutions as taught by the present application (Fig. 6 and 7; see above).

The subject-matter of claim 1 therefore differs from the teaching of D2 in that the sequence of SEQ ID NO: 1 carries the 71 modified codons as taught at pages 9 and 10, i.e. bearing additional silent substitutions.

The problem to be solved by the present invention may therefore be regarded as the provision of a further optimized hG-CSF leading to a higher expression when compared to that obtained with the existing mutants, i.e. at least 50%.

At the time present, inventive activity cannot be acknowledged for the compound of SEQ ID NO: 1 for the following reasons: (I) The present specification teaches the optimization of the hG-CSF sequence by the mutations at positions Arg148, Gly150 (second optimization step, page 15), Ile96 and Gly101 (introduction of the ApaI site) (fourth optimization step, page 16). The obtained mutant, however, bears all the mutations taught at pages 9 and 10. In us5840543 (D2), the modification of the positions Ile96, Gly101, Arg148 and Gly150 were already taught. Should the critical substitutions be those taught at pages 13-16, then no inventive activity can be

recognised since these substitutions were already described in D2, and the compounds of D2 would intrinsically have the claimed technical effect. (ii) it is not clear from the present application which substitutions provide the claimed technical effect. (iii) The expression levels of the mutant of SEQ ID NO: 1 is not compared to that of the already known mutants of hG-CSF. Concerning this, in D3, it is noteworthy that the same expression strain was used (BL21) and that the expression was also induced with IPTG, leading to an expression level of 48% of total E. coli proteins (thus not far from 50%) but using a different culture medium. Concerning this, the same construct bearing SEQ ID NO: 1 (Ftop5), in the same production strain leads to different hG-CSF contents (in % of total proteins) depending on the medium used (see table 1, page 19). Thus, the technical effect of producing at least 50% of the total proteins in E. coli does not seem to be related to the sequence of the claimed construct alone but to a combination of the optimized hG-CSF sequence with the expression vector and the culture conditions.
Since only a comparison is performed between the mutated and the native hG-CSF, but not to other mutated version of the hG-CSF cloned in the same expression vector and cultivated in the same conditions/medium as in the present application, no conclusion can be drawn that the particular high expression is related to the sequence of the Ftop5 construct alone.

The present mutant of SEQ ID NO: 1 thus does not show any inventive activity, since the present nucleotide sequence of SEQ ID NO: 1 does not provide any surprising effect or advantage when compared to the mutants already known from prior art.

2.3 Concerning claim 2, nucleotide sequences which comprise at least one of the combinations listed are already known from prior art but do not lead with the published experimental conditions to the claimed technical effect of an expression of at least 50% of total E. coli protein. It follows that, either this technical effect is not exclusively related to the nucleotide sequence and an objection for lack of complete disclosure of the invention arises, or the already known DNA sequences have intrinsically this characteristic and they would lead to such an expression level under appropriate experimental conditions. In that case, the present application would not bring any contribution to the state of the art.
Relating to this, it is again emphasized that the whole scope claimed must exhibit the inventive step establishing feature, i.e. an expression of at least 50% of total E. coli proteins.

2.4 Consequently, claims 1-24 lack inventive step.

3. Industrial applicability (Art. 33(4) PCT)
The present set of claims shows and industrial application.

4. Additional remarks:

4.1 Present claim 2 (and dependent claims) relates to an extremely large number of possible compounds. Support within the meaning of Art. 6 PCT and disclosure within the meaning of Art. 5 PCT is to be found, however, for only the compound of SEQ ID NO: 1.

4.2 Additional objections for lack of clarity and support by the specification are to be expected for the present set of claims